



Research article

Nitric oxide implication in cadmium-induced programmed cell death in roots and signaling response of yellow lupine plants

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ARTICLE INFO

Article history:

Received 6 February 2012

Accepted 20 June 2012

Available online 4 July 2012

Keywords:

Cadmium

Lupine

Nitric oxide

Programmed cell death

Reactive nitrogen species

Reactive oxygen species

Stress response

ABSTRACT

The sequence of events leading to the programmed cell death (PCD) induced by heavy metals in plants is still the object of extensive investigation. In this study we showed that roots of 3-day old yellow lupine (*Lupinus luteus* L.) seedlings exposed to cadmium (Cd, 89 μ M CdCl₂) resulted in PCD starting from 24 h of stress duration, which was evidenced by TUNEL-positive reaction. Cd-induced PCD was preceded by a relatively early burst of nitric oxide (NO) localized mainly in the root tips. Above changes were accompanied by the NADPH-oxidase-dependent superoxide anion (O₂⁻) production. However, the concomitant high level of both NO and O₂⁻ at the 24th h of Cd exposure did not provoke an enhanced peroxynitrite formation. The treatment with the NADPH-oxidase inhibitor and NO-scavenger significantly reduced O₂⁻ and NO production, respectively, as well as diminished the pool of cells undergoing PCD. The obtained data indicate that boosted NO and O₂⁻ production is required for Cd-induced PCD in lupine roots. Moreover, we found that in roots of 14-day old lupine plants the NO-dependent Cd-induced PCD was correlated with the enhanced level of the post-stress signals in leaves, including distal NO cross-talk with hydrogen peroxide.

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1. Introduction

The response of most plants exposed to toxic environmental factors is usually connected with cell death and organ necrosis. It is frequently difficult to determine whether the observed death is of destructive character, manifested in an uncontrolled spread, or rather it is an effect of programmed and controlled cell self-destruction, generally known as programmed cell death (PCD). This genetically determined process occurs at all stages of the plant life cycle, starting from seed development and germination [1,2] and ending with senescence [3]. PCD events are also involved in plant-biotrophic and plant-necrotrophic [4] pathogen interactions, plant–plant allelopathic interactions [5], hypoxia [6], heat shock [7], as well as a variety of stresses, i.e. ozone [8], ultraviolet [9], salinity [10] and heavy metals stress [11–15].

Cadmium (Cd) as an important environmental pollutant is a non-redox heavy metal, toxic to living organisms. Because of its

high solubility in water it is rapidly taken up by plant roots, in which the most evident phytotoxic symptoms are observed [16]. Simultaneously, plant roots represent the main entry pathway of Cd into the food chain, causing serious problems to human health [17]. Cd pollution disturbs crucial physiological processes in plants, including uptake and transport of water and nutrients, nitrogen metabolism, photosynthesis and respiration [16]. Cd also constitutes a potent inducer of cell death in plant roots. Depending on the metal concentration in the medium diverse forms of cell death may be observed, ranging from apoptosis to necrosis. In confirmation of this statement, in tobacco culture lower concentrations of CdSO₄ (50–100 μ M) induced apoptotic hallmarks, including DNA fragmentation into oligonucleosomal units, while high doses (1000 μ M) showed strong cytotoxicity without DNA programmed fragmentation [18]. Also in onion root apical cells Cd induces apoptosis in a dose-dependent manner and at the concentration above a threshold level of the heavy metal (25–50 μ M CdSO₄), cells display necrosis connected with cell lysis and leakage [11]. The character of cell death mediated by Cd seems to be also cell cycle phase-specific. In tobacco BY-2 cells the application of Cd in the S and G2 phase resulted in apoptosis-like PCD, whereas other cell

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death types, not accompanied by DNA fragmentation, were observed when Cd was applied to cells in the M and G1 phases [19].

According to De Michele et al. [13], the Cd-induced PCD observed in *Arabidopsis* cell cultures resembled an accelerated senescence, since the expression of the senescence-associated gene 12 (*SAG12*), considered as a molecular marker of senescence, preceded cell death. In turn, in tobacco BY-2 cells exposed to Cd, PCD symptoms were accompanied by an increasing expression of the *Hsr203J* gene, an early marker for hypersensitive response (HR) or related types of PCD [14]. In a tomato culture Cd-mediated cell death exhibits morphological and biochemical similarities to plant hypersensitive response, since the metal-induced cell death involves caspase-like proteases, cysteine and serine peptidases, calcium influx, oxidative stress and ethylene accumulation [12].

A growing body of evidence suggests that plant exposure to Cd can induce an increase in signaling compounds, including jasmonate, abscisic acid, ethylene and salicylic acid (SA) [20–22], and it may modulate the time and intensity of nitric oxide (NO) generation as well [22,23]. Nitric oxide is a gaseous free radical molecule involved in many plant growth and development processes, as well as the regulation of multiple responses to biotic and abiotic stress factors [for review, see e.g. 24–26].

Although the formation of NO has been well documented in *in vivo* experiments of various plant systems challenged by Cd, the functional role of endogenous NO during plant response to the heavy metal seems to be much more puzzling if we consider the first results obtained with exogenous NO [27]. Recent reports clearly evidenced that NO contributes to Cd toxicity and the generation of NO might be strictly related to iron deficiency caused by the toxic metal [23,28]. It was revealed in *Arabidopsis* roots that NO initiates the Fe-starvation pathway, promoting the up-regulation of expression of iron acquisition-related genes (*IRT1*, *FRO2* and *FIT*) and in consequence amplifying Cd root accumulation and a subsequent inhibition of root growth [23]. In barley root tips NO was implicated in the metal toxicity mechanism through ectopic and accelerated differentiation, causing a shortening of the root elongation zone and leading in consequence also to root growth inhibition [29]. Similarly, in wheat plants Cd-induced NO formation was directly correlated with root growth inhibition [30]. In an *Arabidopsis* culture an elevated NO production reduced the efficiency of Cd ion detoxication through direct S-nitrosylation of phytochelatin, promoting deleterious effects of Cd including the active cell death [13].

Apart from NO a prominent role in PCD events is played by reactive oxygen species (ROS), which is reliably evidenced for HR cell death [31–33]; however, the molecular mechanism of the interaction between both ROS and reactive nitrogen species (RNS) during the execution of the cell death program is still far from being clarified. In the opinion of Zottini et al. [34], NO might induce cell death by interfering with mitochondria functionality, which can have a significant impact on the cellular balance between ROS generation and scavenging.

Recently, several lines of evidence have highlighted the role of NO in the modulation of Cd-induced cell death in cell suspension systems [13,14], but exactly how NO cooperates with ROS to trigger PCD in intact plants exposed to heavy metals is still the object of extensive investigations.

In this study we addressed the questions of NO implication in cadmium-induced cell death of lupine roots. We determined the character of dying out of root cells, supported by TUNEL reaction and evaluated the degree of DNA fragmentation of the damaged cells by comet assays in response to Cd ions. In particular, we examined NO generating and scavenging systems in cooperation with ROS involvement to trigger active cell death mediated by cadmium. Finally, the correlation between PCD symptoms observed in roots and the level of the post-stress signal generation, including NO

orchestration with hydrogen peroxide (H_2O_2) and SA was evidenced in lupine leaves, proving that lupine roots through Cd-challenged PCD may generate a mobile long-distance signal from roots to leaves.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of yellow lupine (*Lupinus luteus* L. cv. Ventus) were surface-sterilized with 75% (v/v) ethanol and 1% sodium hypochlorite and washed thoroughly several times with distilled water. Seeds germinated in Petri dishes containing water-moistened filter paper for 48 h in the dark at $22 \pm 1^\circ\text{C}$.

For experiments on 3-day old plants, seedlings were transferred to dishes containing 4 ml of either distilled water (control) or depending on the experiments: 100 μM PTIO (2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide), 50 μM DPI (diphenyleneiodonium chloride), 100 μM SIN-1 (3-morpholiniosydnonimine hydrochloride), 50 μM ebselen, 89 μM CdCl_2 (which corresponds to 10 mg l^{-1} of Cd), 89 μM CdCl_2 + 100 μM PTIO, 89 μM CdCl_2 + 50 μM DPI, 89 μM CdCl_2 + 100 μM SIN-1, 89 μM CdCl_2 + 50 μM ebselen or 223 μM CdCl_2 (which corresponds to 25 mg l^{-1} of Cd). Seedlings were incubated in the dark at $22 \pm 1^\circ\text{C}$ up to 24 h. Depending on the experiment the material was analyzed at 2, 6, 12 and 24 h of incubation. Concentrations of Cd were established experimentally (see Supporting information Fig. S1).

For the experiment on 12-day old plants, seedlings were transferred to a modified culture system as described by Przymusiński et al. [35], composed of filter paper soaked with H_2O placed within plastic growth pouches. The seedlings were grown for 9 days at $22 \pm 1^\circ\text{C}$ with an 18/6 h photoperiod and light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. At the stage of the second fully expanded leaf seedlings were treated at the root level either with distilled water (control) or 89 μM CdCl_2 for 24 h and 48 h.

2.2. NO-donor treatment

To evaluate the effect of exogenous NO on programmed cell death the seedlings of lupine were transferred onto Petri dishes and incubated for 5 h in 50 μM SNAP (S-nitroso-N-acetyl-DL-penicillamine) under continuous light conditions according to Floryszak-Wieczorek et al. [36].

2.3. TUNEL assay

The assay measures DNA fragmentation using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end-labeling method, in which TdT incorporates fluorescein-12-dUTP on the 3'-OH ends of fragmented DNA (TUNEL fluorescein, Roche, Indianapolis, USA). An antifuorescein-alkaline phosphatase conjugate (TUNEL AP, Roche, Indianapolis, USA) subsequently binds dUDP-fluorescein, and in the presence of Fast Red (Roche) shows red precipitate forms in the nuclei containing fragmented DNA. Thin slices of the root tissues were immersed for 1 h in 4% formaldehyde in phosphate buffered saline (PBS). After being rinsed in PBS, the samples were treated with liquid nitrogen. The samples were rehydrated and the TUNEL reaction was performed according to the manufacturer's protocol. Negative controls were conducted in the absence of the TUNEL enzyme. In positive controls prior to labeling the tissue was incubated with DNase I (Roche) for 10 min at 25°C . The samples were examined under a light microscope (TUNEL AP) or a fluorescence microscope (TUNEL fluorescein) (Axiostar plus Carl Zeiss, Jena, Germany) equipped with a digital camera at excitation of 488 nm and emission of 515 nm. Experiments were repeated three times

with ten slices per treatment. A region of 100 cortex cells from at least 5 randomly selected slides in each treatment was counted and statistically analyzed.

2.4. Comet assay

The DNA damage was determined by an alkaline comet assay, according to the method described by Olive and Banath [37]. Nuclei of the lupine root cells were isolated and electrophoresed as described in details by Rucińska et al. [38]. Slides were neutralized (400 mM Tris; pH 7.5), then washed with water, 70% ethanol and dried at room temperature. Immediately prior to scoring the DNA was stained with 300 μ l SYBR Green I (Molecular Probes Invitrogen, Eugene) (dilution 1: 10 000). The comets were observed using a confocal fluorescence microscope (LSM 510 Carl Zeiss, Jena, Germany) equipped with an excitation filter of 500–550 nm and a barrier filter of 590 nm. The extent of DNA migration was determined using the CASP-image analysis system (University of Wrocław, Poland). Tail length, one of the DNA damage parameters, is positively correlated with the level of DNA breakage and/or alkali-labile sites. Because the measurement system was not calibrated, tail length was expressed in arbitrary units. Generally 150 comets were scored per dose (50 comets were randomly selected from three replicated slides).

2.5. NO detection by confocal laser scanning microscopy

NO formation was detected using a fluorescent DAF-2DA dye (Calbiochem) according to Floryszak-Wieczorek et al. [4]. A Zeiss Axiovert 200 M inverted microscope equipped with a confocal laser scanner (LSM 510 Carl Zeiss, Jena, Germany) was used in this study and sections were excited with the 488 nm line of an argon laser. Dye emissions were recorded using a 505–530 nm band-pass filter and the autofluorescence of chloroplasts was captured with a 585 nm long-pass filter. Microscope, laser and photomultiplier settings were held constant during the experiment in order to obtain comparable data. Images were processed and analyzed by Zeiss LSM 510 software. As found Arita et al. [39] NO-dependent fluorescence is not observed only in the presence of high concentrations of the specific NO-scavenger (peak absorption wavelengths overlap with the DAF-2T fluorescence emission spectrum), therefore in this experiment 1 mM PTIO was used.

2.6. NADPH-oxidase activity assay

In order to assay plasma membrane-bound NADPH activity, plasma membranes of root tips were isolated as described by Sagi and Fluhr [40]. The protein content of the membrane fraction was estimated according to Bradford [41], using bovine serum albumin as a standard. NADPH-oxidase activity was assayed spectrophotometrically by the change in absorbance (A_{470}) yielded by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) reduction, according to Sagi and Fluhr [40].

2.7. Measurement of ROS production

The level of $O_2^{\cdot -}$ was assayed spectrophotometrically on the basis of the capacity of the superoxide anion radical to reduce nitro blue tetrazolium (NBT) to diformazan, according to Doke [42]. H_2O_2 concentration was precisely determined spectrophotometrically using the titanium (Ti^{4+}) method [43]. Additionally, H_2O_2 was assayed using the cytochemical detection method by a color reaction with 3,3'-diaminobenzidine (DAB), according to Thordal-Christensen et al. [44].

2.8. Detection of peroxynitrite

The level of peroxynitrite was assayed according to Huang et al. [45] using folic acid as the peroxynitrite scavenger, giving high fluorescent emission products. Root tips (1 g) were immersed in the incubation mixture, containing a barbital buffer solution (pH 9.4) and folic acid (1.0×10^{-5} mol l^{-1}). Fluorescence intensity of the solution was recorded at 460 nm with the excitation wavelength set at 380 nm. The standard curve was prepared for SIN-1 (Calbiochem) as a donor of peroxynitrite at the range of concentrations from 1 to 14 nM.

2.9. Measurement of cell viability

Cell viability, indicated as a loss of plasma membrane integrity, was measured spectrophotometrically as the Evans Blue uptake [46].

2.10. Determination of Cd content

Cadmium content in roots was determined after a prior mineralization using an atomic spectrometer. Cadmium was also detected histochemically according to the procedure described by Seregin and Ivanov [47] using dithizone (diphenylthiocarbazone, 30 mg dissolved in 60 ml acetone and 20 ml distilled water). The intensification of a brown coloring corresponds to Cd accumulation.

2.11. Determination of salicylic acid level

Measurement of SA content essentially followed the protocol of Meuwly and Métraux [48]. HPLC analysis was performed on the Agilent HP 1100 HPLC system, equipped with a photodiode array detector (DAD). A Lichrospher 100 RP18 column (250.0×4.0 mm, 5.0μ m) by Merck was used for all separations. The mobile phase consisted of water, methanol and acetic acid (69:28:3, v/v/v), applied in the isocratic elution. The flow rate was adjusted to 1 ml min^{-1} , the detection wavelength was set to DAD at $\lambda = 302$ nm and 40 μ l of samples were injected. All separations were performed at the temperature of 45 °C. Peaks were assigned by spiking the samples with the standard compound and a comparison of the UV-spectra and retention times ($t_R = 8$ min). The analytical method was validated according to ICH guidelines. The relationship between the peak area and the concentration of salicylic acid injected was found to be highly linear with a regression coefficient $R^2 > 0.9999$. Intra-day repeatability of the HPLC–DAD method was evaluated by performing five repetitive analyses of the standard, which gave an RSD 2 of 15%, showing good precision.

2.12. Statistical analysis

All results are based on three independent experiments, each with at least three biological replicates. For each experiment means of the obtained values were calculated along with standard deviations. The analysis of variance was conducted and the least significant differences (LSDs) between means were determined using Tukey's test at the level of significance $\alpha = 0.05$ and $\alpha = 0.01$. In case of comet assay the Kruskal–Wallis analysis of variance test with nonparametric distribution was used and statistical significance was considered at $\alpha = 0.01$.

3. Results

3.1. NO-mediated PCD challenged by Cd

Lupine root cells exposed to 24 h Cd stress exhibited symptoms of active death, which was found in the TUNEL-positive test

illustrating the internucleosomal fragmentation of DNA in cortex cells of the root elongation zone (Fig. 1(a)). Based on the number of TUNEL-positive nuclei we found that the effect was dose-dependent as a lower Cd concentration (89 μM CdCl_2) promoted active death of root cells in about 30% and treatment with a very high metal dose (223 μM CdCl_2) resulted in only 14% of TUNEL-positive nuclei, respectively (Fig. 1(d)). In order to verify whether NO is capable of modifying the response of lupine seedling roots to Cd stress, the NO-donor in the form of 50 μM SNAP and the NO-scavenger as 100 μM PTIO were applied. As it was shown in Fig. 1(d), supplementation of root cells with NO did not result in significant increase in the number of TUNEL-positive tests. However, the elimination of endogenous NO caused a percentage decrease from 30% to only 14% under 89 μM CdCl_2 exposure. Inhibition of NADPH-oxidase-dependent O_2^- accumulation by DPI (diphenyleneiodonium chloride) application reduced the pool of TUNEL-positive cells to 12% as well. Treatment of control lupine roots with all chemicals (i.e. SNAP, PTIO, DPI) had no significant effect on the observed number of TUNEL-positive nuclei.

Additionally, we used the alkaline comet assay to evaluate the degree of DNA damage in lupine roots undergoing Cd stress (Supporting information Fig. S1). Fig. S1a shows an individual isolated nucleus comprising a head and a tail forming a comet image. The extent of DNA damage within the population of Cd-affected nuclei (expressed as a tail length) was intensified at both tested Cd concentrations (Supporting information Fig. S1a and S1b). The elimination of endogenous NO (100 μM PTIO) during Cd exposition provoked an augmentation of the analyzed parameter, however this increase was statistically insignificant.

3.2. RNS and ROS generation during Cd-induced PCD

Since we found that a lower Cd dose promotes PCD, therefore only the metal concentration of 89 μM CdCl_2 was used in the following experiments. Simultaneously, 89 μM CdCl_2 includes in the tolerance limit noted for yellow lupine seedlings, since the inhibition of root growth by 50%, known as tolerance index [49], was observed at Cd concentration 134 μM (Supporting information Fig. S2).

Real-time imaging of NO production in 3-day old lupine root seedlings was performed by specific fluorochrome DAF-2DA. Green fluorescence of the triazole molecule (DAF-2T) formed from

DAF-2DA, indicating the presence of endogenous NO, was found in non-stressed seedling roots in the apical zone of roots (Fig. 2(a) and (b)). Initially, root tips during the first 6 h exposure to cadmium did not show well-defined changes in NO production (Fig. 2(c) and (d)) and only progressive heavy metal stress provoked a significant increase in NO-dependent fluorescence, which spread over the epidermal and external layers of cortex cells (Fig. 2(e)–(h)). The highest NO accumulation was detected at 24 h of stress duration and the observed NO-dependent fluorescence was almost totally scavenged by 1 mM PTIO application (Fig. 2(i) and (j)). DPI treatment had no effect on the Cd-induced NO production (Fig. 2(i) and (j)).

In lupine roots treated with 89 μM CdCl_2 NADPH-oxidase activity started to increase after 12 h of stress duration and the most impressive rise of the activity by ca. 40% was recorded at the 24th h (Fig. 3). Since NADPH-oxidase is DPI-sensitive, therefore an addition of 50 μM DPI resulted in a suppression of the enzyme activity mainly at 24 h.

An increased NADPH-oxidase activity caused by Cd stress was correlated in time with superoxide accumulation. The O_2^- production increased almost two-fold in response to Cd at 24th h (Fig. 4(a)). DPI treatment significantly reduced O_2^- synthesis, to evidence NADPH-oxidase as the main source of ROS in lupine roots under short-term Cd stress. Elimination of NO during 24 h of Cd stress did not affect NADPH-oxidase activity as well as superoxide accumulation (Figs. 3 and 4(a)). In turn, the dynamics of H_2O_2 accumulation under the influence of Cd ions showed only a slight increase in the level of this compound during the first 24 h of stress (Fig. 4(b)).

The concomitant high level of both NO and O_2^- observed in lupine root cells at 24 h of Cd exposure did not augment ONOO[−] generation (Fig. 5(a)). Importantly, non-stressed control roots showed relatively high constitutive levels of ONOO[−] in comparison to the metal stressed ones. The application of both the specific NO (PTIO) and ONOO[−] (ebselen) scavengers, resulted in the suppression of peroxynitrite generation. In turn, SIN-1, as an ONOO[−] donor which gradually decomposes to yield equimolar amounts of NO and O_2^- , caused a significant increase in ONOO formation in both control and Cd stressed roots. Since there is evidence [31] that ONOO[−] is not involved in NO-mediated cell death in plants, we also examined the effect of peroxynitrite on the cell viability of lupine roots. As it had been expected, scavenging of endogenous ONOO[−] in control and

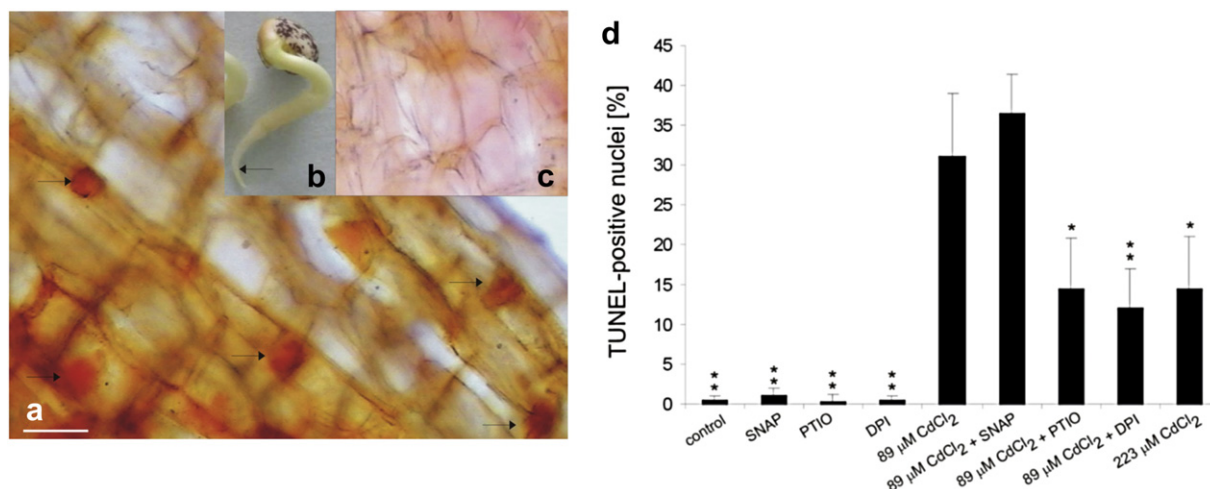


Fig. 1. Identification of cell death in lupine root seedlings by TUNEL AP assay. A representative image of TUNEL-positive nuclei of root cortex cells exposed for 24 h to 89 μM CdCl_2 , arrows indicate TUNEL-positive nuclei (a); the part of the root taken for the analysis (b); TUNEL-negative control (c). The percentage of cortex cells exhibiting TUNEL-positive reaction after following treatments: H_2O – control, 50 μM SNAP, 100 μM PTIO, 50 μM DPI, 89 μM CdCl_2 , 89 μM CdCl_2 + 50 μM SNAP, 89 μM CdCl_2 + 100 μM PTIO, 89 μM CdCl_2 + 50 μM DPI, 223 μM CdCl_2 (d). 100 Cortex cells from at least 5 randomly selected slides were examined in each treatment. Asterisks indicate values that differ significantly from the 89 μM CdCl_2 treated lupine root seedlings at $P < 0.05$ (*) and $P < 0.01$ (**). Bar = 8 μm .

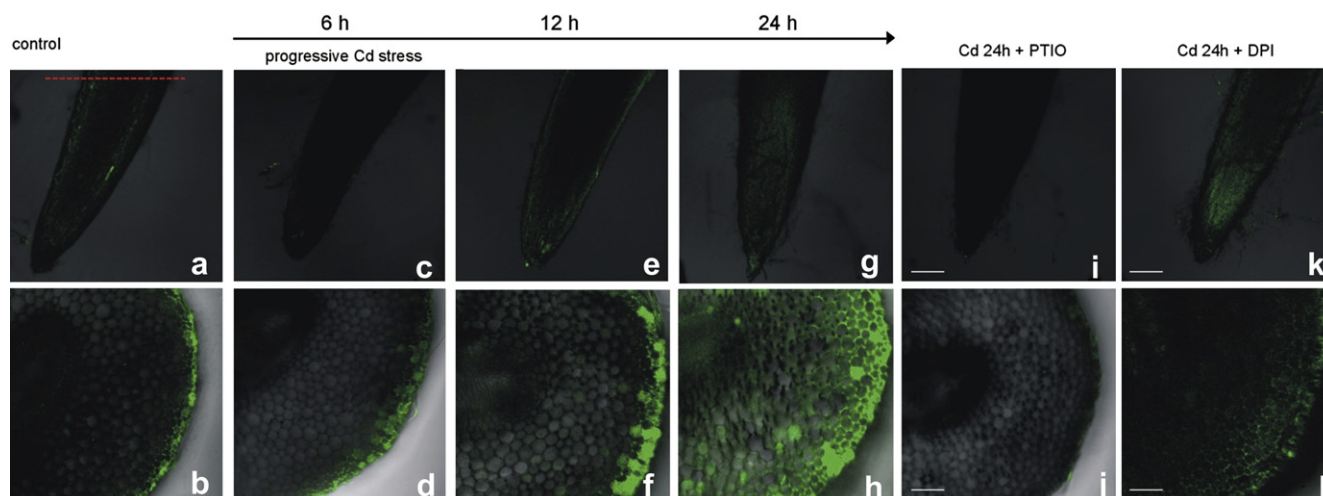


Fig. 2. Bio-imaging of nitric oxide generation with DAF-2DA fluorescent probe in lupine root seedlings. NO was analyzed in non-stressed control root seedlings (a, b) as well as at 6 h (c, d), 12 h (e, f), 24 h (g, h) after seedlings exposure to 89 μM CdCl_2 . NO detection at 24 h after treatment with 89 μM CdCl_2 + 1 mM PTIO (i, j) and 89 μM CdCl_2 + 50 μM DPI (k, l); lower panel corresponds to cross-sections obtained from the zone indicated with red line. Images show general phenomena representative of three individual experiments. Bars indicate, a, c, e, g, i, k: 300 μm ; b, d, f, h, j, l: 100 μm .

during heavy metal exposure did not modify cell viability (Fig. 5(b)). The sequential treatment with Cd ions and SIN-1 did not increase the number of dying cells. Surprisingly, we observed that ONOO⁻ being released during Cd stress was even favorable to lupine roots cell viability. A similar effect we noted in Cd and PTIO treated seedling roots. However, SIN-1 and PTIO application to control seedlings did not modify the viability of lupine root cells.

3.3. NO affects Cd accumulation in lupine root cells

Treatment at a dose of 89 μM CdCl_2 resulted in Cd accumulation in lupine root tissues amounting to $0.217 \pm 0.049 \text{ mg g}^{-1} \text{ DW}$ at 24 h (Fig. 6(a)). Limitation of endogenous NO, owing to the application of 100 μM PTIO, showed ca. 54% reduction of Cd content. Additionally, the occurrence of Cd in lupine root tips was also visualized using dithizone-dependent staining, where reddish-brown coloring correlated with Cd localization (Fig. 6(b)–(d)). In this way we found that the reduction of Cd content related to NO scavenging by PTIO application was especially evident in cells of the cortex and the vascular cylinder (Fig. 6(d)).

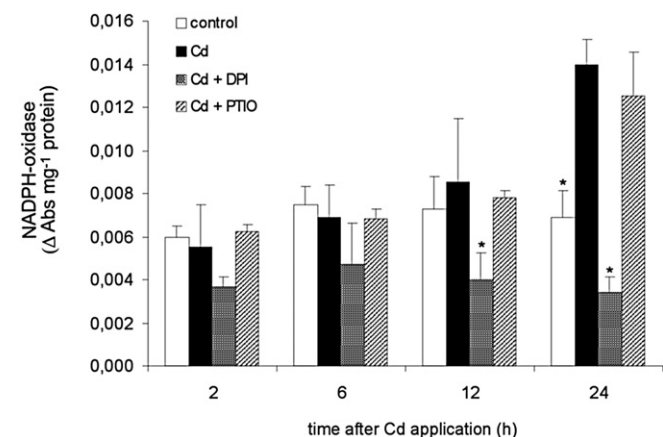


Fig. 3. The effect of progressive cadmium stress supplied as 89 μM CdCl_2 , 89 μM CdCl_2 + 50 μM DPI and 89 μM CdCl_2 + 100 μM PTIO on plasma membrane-bound NADPH-oxidase activity in roots of lupine seedlings. Asterisks indicate values that differ significantly from the 89 μM CdCl_2 treated lupine root seedlings at $P < 0.05$ (*).

3.4. The effect of Cd on post-stress signaling molecules in roots and leaves of 14-day old lupine seedlings

To determine if roots exposure to Cd may modulate post-stress signaling network in leaves, another experimental design was used

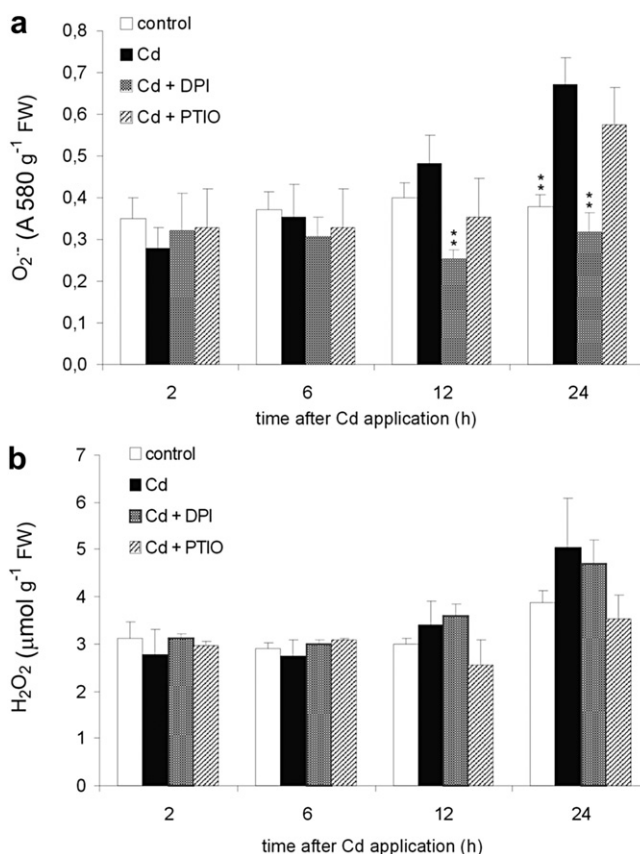


Fig. 4. The effect of progressive cadmium stress in lupine seedling roots supplied as 89 μM CdCl_2 , 89 μM CdCl_2 + 50 μM DPI or 89 μM CdCl_2 + 100 μM PTIO on superoxide generation (a); and hydrogen peroxide accumulation (b). Asterisks indicate values that differ significantly from the 89 μM CdCl_2 treated lupine root seedlings at $P < 0.01$ (**).

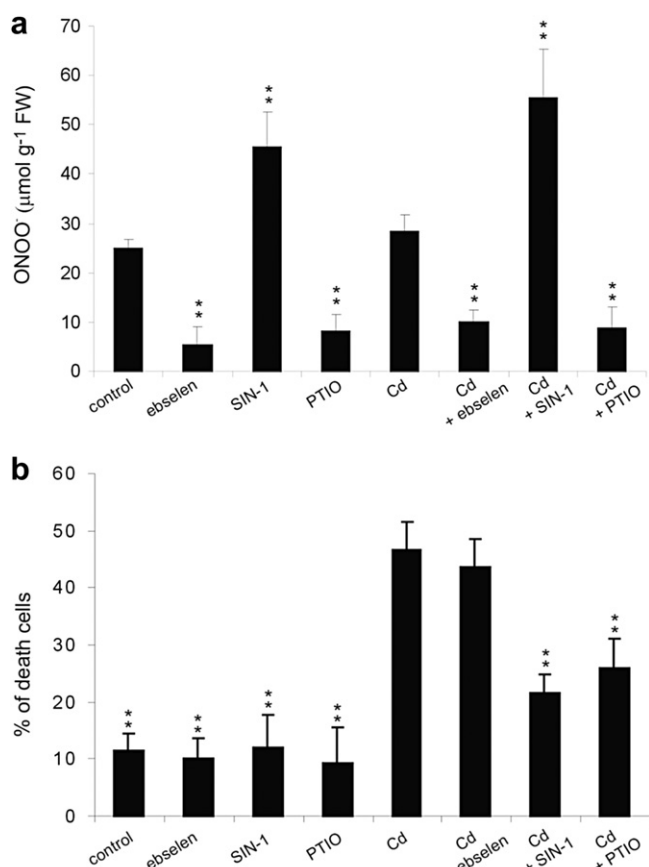


Fig. 5. Peroxynitrite generation (a); and cell viability tested by Evans Blue uptake (b); in lupine roots at 24 h after following treatment: H₂O (control), 50 μM ebiselen, 100 μM SIN-1, 100 μM PTIO, 89 μM CdCl₂, 89 μM CdCl₂ + 50 μM ebiselen, 89 μM CdCl₂ + 100 μM SIN-1 or 89 μM CdCl₂ + 100 μM PTIO. Asterisks indicate values that differ significantly from the 89 μM CdCl₂ treated lupine root seedlings at $P < 0.01$ (**).

with 14-day old lupine seedlings at the stage of two fully expanded leaves. Based on DAF-2T fluorescence we observed that control plants generated some amount of NO only in the root apical zone (Fig. 7(j)). In turn, the treatment with 89 μM CdCl₂ for 24 h visibly triggered NO synthesis in the elongation and differentiation zone of lupine roots (Fig. 7(f)–(h)) as well as in leaves (Fig. 7(a)–(c)). The green NO-dependent fluorescence in leaves was found particularly in vascular bundles and surrounding cells (Fig. 7(a)–(c)). The application of 1 mM PTIO almost completely eliminated green fluorescence in Cd-treated roots and leaves as well (Fig. 7(d) and (i)).

When comparing the stress signaling system between plant organs it was found that Cd-induced NO synthesis was not accompanied by statistically significant H₂O₂ accumulation in lupine roots, while in leaves an approximately two-fold increase in H₂O₂ was tuned with NO-dependent compounds (Fig. 8(a)). An additional histochemical analysis revealed that H₂O₂, similarly as the NO signal, was localized mainly in leaf vascular bundles (Fig. 8(c) and (d)).

The short-term exposure of lupine seedlings to Cd (24 h) provoked a slight increase in free SA in leaves (Supporting information Fig. S3). Interestingly, the application of the NO-scavenger during Cd stress prevented not only H₂O₂ accumulation but also lowered the SA synthesis in lupine leaves (Fig. 8(e), Supporting information Fig. S3).

In order to investigate whether Cd-induced over-accumulation of post-stress signaling molecules in lupine leaves could be

attributed to PCD events observed in roots of 14-day old lupine seedlings we additionally performed the TUNEL fluorescein assay. As it was shown in Fig. 9(a)–(c) and (e), Cd-exposed root cells exhibited a TUNEL-positive reaction starting after 24 h.

4. Discussion

Cadmium pollutions are well-known to disturb the crucial physiological processes in plants, resulting in a variety of toxicity symptoms ranging from chlorosis, growth inhibition to cell death. Results of this study demonstrate that Cd reduced root growth of lupine seedlings, and at 89 μM CdCl₂ root cells undergo PCD with TUNEL-positive nuclei (Fig. 1). The typical hallmarks of PCD upon Cd treatment were observed earlier in cell suspensions of tobacco [18,19,14], tomato [12], white poplar [50], *Arabidopsis* [13], as well as onion root apical cells [11], in root tip and leaf tissue of *Genipa americana* [15]. Some reports indicated a critical dose-dependent Cd exposure, below which PCD will be triggered and above which necrotic cell death will dominate [18,11]. Our results confirm the existence of a threshold for PCD in Cd-challenged plants, since 89 μM CdCl₂ triggered active death exhibited as a TUNEL-positive reaction, while a higher Cd concentration (223 μM CdCl₂) significantly reduced the number of TUNEL-positive nuclei. The limit value of Cd seems to be strictly dependent on the experimental designs, i.e. metal forms and concentrations, duration of stress treatment, plant species and developmental phases of model plants.

NO has been shown to be involved in the regulation of mechanisms controlling the growth and developmental processes of plant roots [51]. Also in our study the specific green fluorescence of the triazole molecule (DAF-2T) formed from DAF-2DA was recorded in non-stressed lupine root tips, which was probably linked with the signaling mode of NO, indirectly driving root morphological processes (Fig. 2(a) and (b)).

Cd-induced PCD in lupine roots was preceded by a relatively early burst of NO. The DAF-2DA fluorescence microscopy revealed enhanced NO generation in the apical zone of roots in response to the heavy metal at 12 h of stress duration (Fig. 2(c)–(h)). Cd was able to trigger NO synthesis within the first several hours of stress exposure in roots of other plant species as well [23,29]. However, there are still conflicting data regarding the time and intensity of NO generation in response to Cd. For instance, in rice roots a 24-h treatment with 100 μM CdCl₂ significantly reduces NO accumulation correlated with the inhibition of NOS-like activity [52]. Similarly, a relatively short (48 h) exposure to 50 μM CdSO₄ considerably reduces NO accumulation in *Medicago truncatula* roots [53]. Also a long-term cadmium stress (14d, 50 μM CdCl₂) strongly diminished NO content in pea roots, which was especially evident in cell walls of cortex cells [54].

Importantly, we demonstrated that scavenging of the endogenous NO pool during Cd stress reduced the number of the TUNEL-positive nuclei (Fig. 1), however the lack of NO did not significantly affect the degree of DNA damage evidenced by comet assay (Supporting information Fig. S1). The data indicate that the boosted NO production within the first hours of Cd stress is required to initiate the active cell death program in lupine roots.

An increase in NO accumulation in response to Cd was accompanied by PCD occurring in tobacco BY-2 cells as well [14]. In an *Arabidopsis* cell suspension the NO production started 24 h after Cd treatment and its level remained high as long as cells were viable. However, cells with PCD symptoms were found as late as the 4th day of metal treatment [13]. Although experiments on cell suspensions are highly informative, data collected using cell cultures do not take into account stress-induced, cross- and long-distance communication between cells and neighboring tissues or

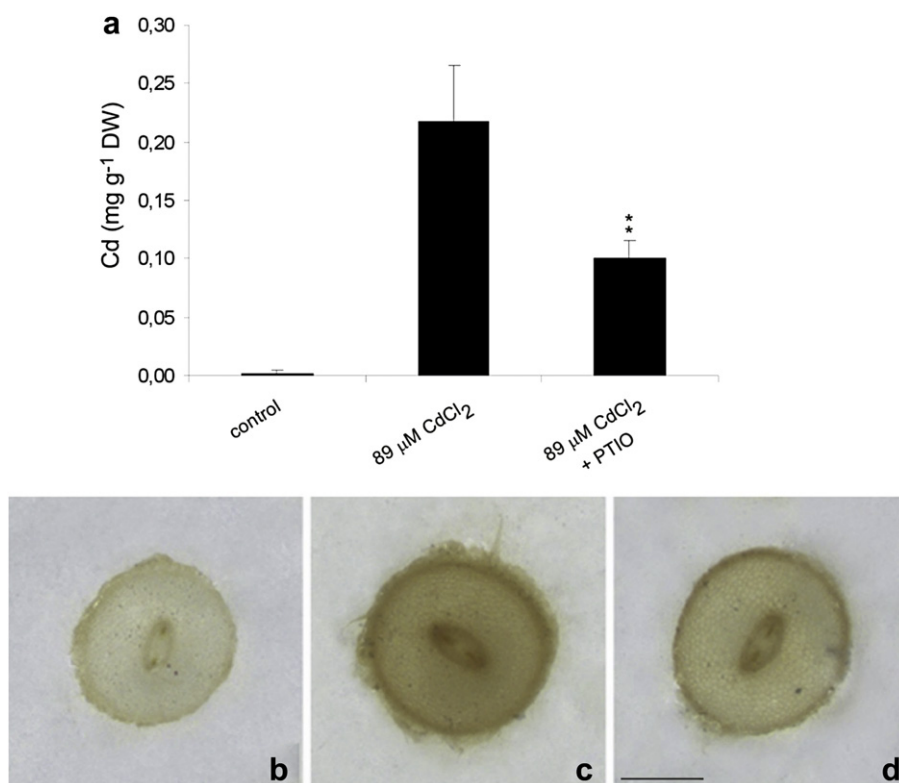


Fig. 6. The effect of endogenous NO depletion on Cd content in root cells of lupine seedlings. Quantification of the Cd content in root seedlings treated with 89 μM CdCl₂ or 89 μM CdCl₂ + 100 μM PTIO for 24 h (a). Visualization of Cd by dithizone complex (diphenylthiocarbazone complex) at cross-sections of control root seedlings (b), as well as seedlings treated with 89 μM CdCl₂ (c) or 89 μM CdCl₂ + 100 μM PTIO (d) at 24 h after metal exposure. Bar: 1000 μm; images show general phenomena representative of three individual experiments. Asterisks indicate values that differ significantly from the 89 μM CdCl₂ treated lupine root seedlings at $P < 0.01$ (**).

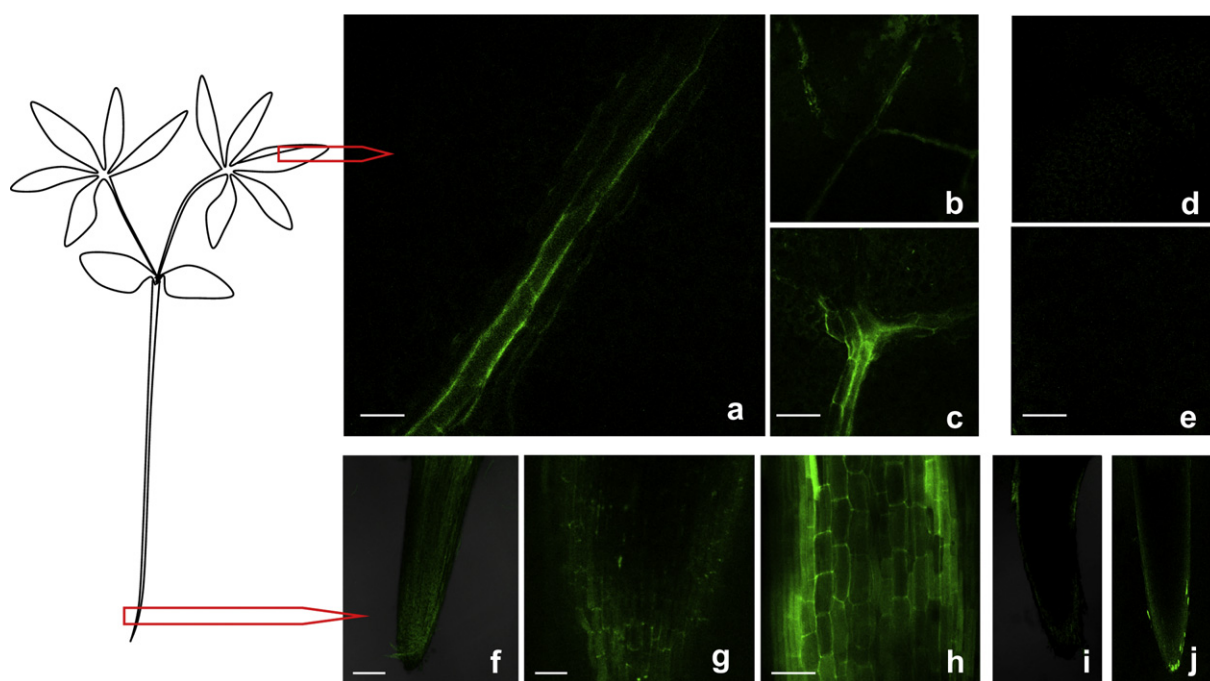


Fig. 7. Bio-imaging of nitric oxide with DAF-2DA fluorescent probe in 14-day old lupine seedlings. NO was analyzed in both leaves (a–c) and roots (f–h) at 24 h after seedling roots exposure to 89 μM CdCl₂; leaves (d) and roots (i) at 24 h after treatment with 89 μM CdCl₂ + 1 mM PTIO; leaves (e) and roots (j) of control plants. On the left, the plant schematic shape shows the site of analysis. Images show general phenomena representative of three individual experiments. Bars indicate, a: 50 μm; b: 200 μm; c–e, g, h: 100 μm; f, i, j: 500 μm.

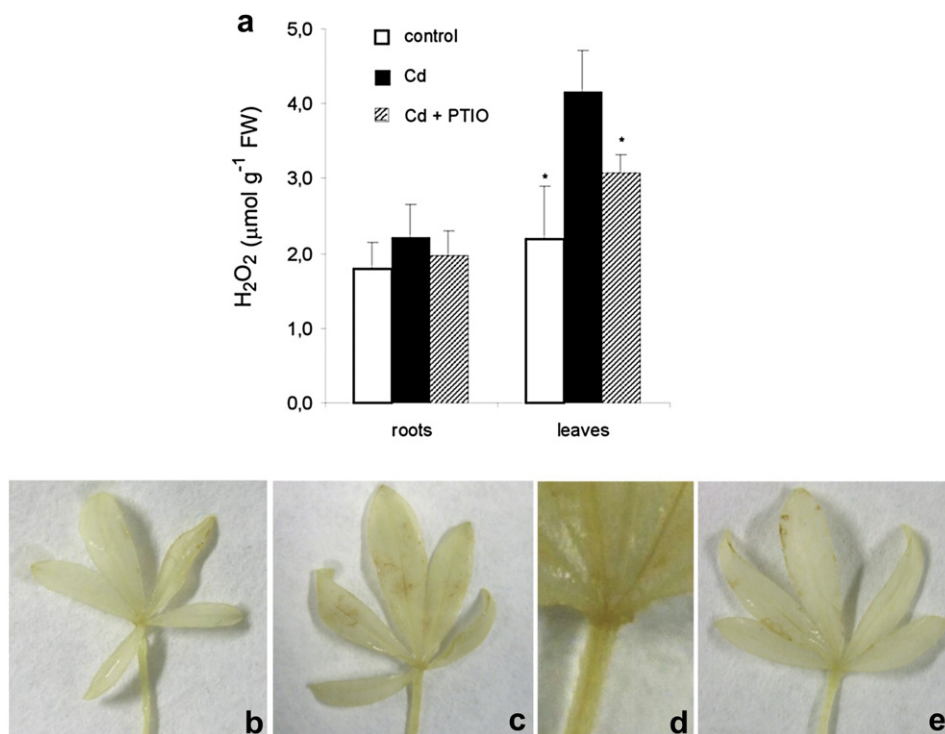


Fig. 8. The level of H_2O_2 in 14-day old lupine seedlings exposed to 89 μM CdCl₂ at 24 h. Spectrophotometric assay of H_2O_2 accumulation (a). Histochemical localization of H_2O_2 in leaves of control (b), 89 μM CdCl₂ (c, d) and 89 μM CdCl₂ + 100 μM PTIO (e) treated seedlings. Asterisks indicate values that differ significantly from the 89 μM CdCl₂ treated lupine seedlings at $P < 0.05$ (*).

organs, which seems to be very important during the heavy metal-induced signaling network, e.g. via NO [27].

It was revealed in many experimental designs using NO-donors that exogenously applied NO can provide plant protection e.g. against heavy metals toxicity; however, in view of the recent reports [23,28] it needs to be asked whether NO generation is really favor to the plant undergoing heavy metal stress. In our conditions, scavenging of the endogenous NO pool reduced the number of TUNEL-positive nuclei (Fig. 1(d)), but also the accumulation of Cd in root cells (Fig. 6). Similarly, supplementation of tobacco BY-2 cells with

NO triggered PCD and increased Cd level within cells [14]. The histological localization of Cd by the dithizone complex in lupine roots showed that trapping of NO by PTIO application reduced the heavy metal accumulation in cells of the cortex and central cylinder. In agreement with Ma et al. [14] the mechanism, by which NO regulates PCD, may be largely due to its ability to modulate Cd entry and accelerate Cd accumulation. It was earlier evidenced that Cd enters root cells through the IRT1 transporter, in turn NO promotes the up-regulation of iron acquisition-related genes expression (*IRT1*, *FRO2* and *FIT*) and in consequence NO amplifies Cd uptake [23].

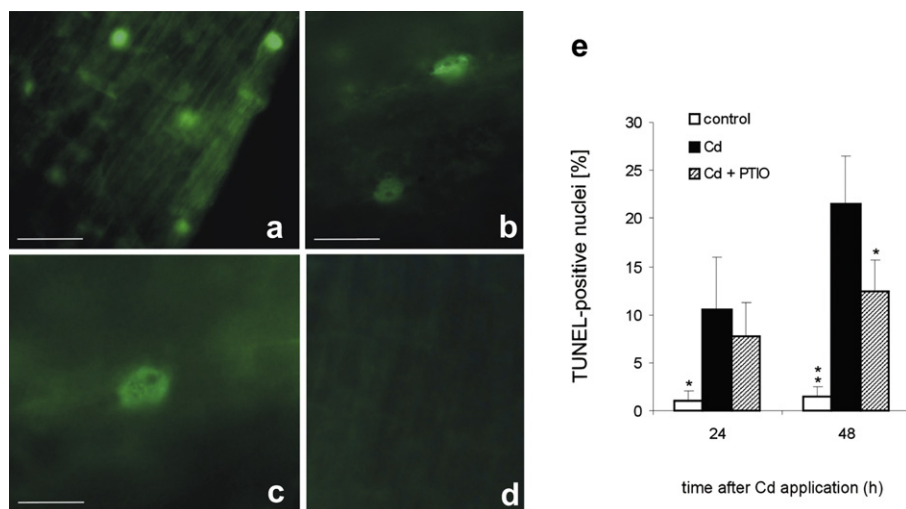


Fig. 9. Identification of cell death in 14-day old lupine root seedlings exposed to 89 μM CdCl₂ by TUNEL fluorescein assay. A representative images of TUNEL-positive nuclei of cortex cells (a–c); TUNEL-negative control (d); bars indicate, a, d: 20 μm ; b, c: 5 μm . The percentage of cortex cells exhibited TUNEL-positive reaction at 24 h and 48 h of 89 μM CdCl₂ stress duration (e). 100 Cortex cells from at least 5 randomly selected slides were examined in each time point per treatment. Asterisks indicate values that differ significantly from the 89 μM CdCl₂ treated lupine root seedlings at $P < 0.05$ (*) and $P < 0.01$ (**).

An early NO generation found in cells of lupine root seedlings exhibiting PCD symptoms was accompanied by the activation of plasma membrane NADPH-oxidase (Fig. 3) and subsequent superoxide anion accumulation (Fig. 4), starting from 24 h after Cd application. It has been documented that apart from oxalate oxidase and extracellular peroxidases [55], the plasma membrane-bound NADPH-oxidase is implicated in the earliest Cd-induced ROS production after a short-term metal stress [56,57].

Treatment with DPI, an NADPH-oxidase inhibitor (Fig. 3), decreased the enzyme activity and significantly reduced O_2^- production (Fig. 4), suggesting that also in our plant model system plasma membrane-bound NADPH-oxidase is engaged in an early oxidative burst mediated by Cd. Simultaneously, the suppression of the NADPH-oxidase-dependent source of O_2^- generation, resulted in a significant reduction of cells undergoing PCD. Our new finding is that a simultaneous enhancement of both the NADPH-oxidase-dependent O_2^- and NO production is essential for lupine root cells to enter the PCD pathway. Several lines of evidence have attempted to explain the interplay between ROS and NO during PCD establishment with the use of different plant model systems [31,32,58–60]. Delledonne et al. [31] presented data that the cell death mechanism during HR is controlled by a balanced production of NO and mainly H_2O_2 among ROS compounds. In soybean and tobacco cell suspensions a simultaneous increase in both NO and H_2O_2 activated cell death, whereas an independent increase in only one of the above mentioned molecules induced death only slightly [61,62]. De Michelle et al. [13] postulated that H_2O_2 accumulation, preceding cell death provoked by Cd by ca. 24 h, is rather a part of the degenerative than a signaling event; however relatively high levels of both H_2O_2 and NO were necessary to trigger PCD in *Arabidopsis* cells. Our time-course experiments indicated that O_2^- rather than H_2O_2 functions as the molecule that synergizes with NO to unlock the PCD program in lupine roots, since the level of H_2O_2 in lupine roots treated with Cd increased only slightly during the first 24 h. Likewise, Zhao et al. [59] showed that both NO and O_2^- were required for elicitor-induced cell death in a *Cupressus lusitanica* cell culture.

Based on APF (aminophenyl fluorescein) fluorescence microscopy, it was recently found that roots of yellow lupine exposed to a short-term (24 h) Cd stress produced ONOO⁻ ions in the differentiation and elongation zone of roots [27]. The fluorometric assay used in this study allowing us to quantitatively show ONOO⁻ generation (Fig. 5(a)); however enhanced level of both parent molecules, i.e. NO and O_2^- noted at 24 h of Cd stress did not significantly increase ONOO⁻ formation in lupine roots. An uniquely early increase of ONOO⁻ dependent fluorescence (at the 2nd h of stress duration) was found in pea roots treated with $CuSO_4$. In contrast to copper treatment, pea roots exposure to 100 μ M $CdCl_2$ for 24 h and 48 h induced NO generation without ONOO⁻ formation [63]. An additional analysis revealed that ONOO⁻ does not affect cell viability in lupine roots (Fig. 5(b)); interestingly, SIN-1 treatment even augmented cell viability under Cd stress. Recently it has been stated that ONOO⁻ might modulate cell viability by resetting the boosted NO signal upon Cd challenge [64]. Moreover, ONOO⁻ formation could provide an important regulatory loop for NO activity under stress conditions, since ONOO⁻ can provoke tyrosine nitration phenomena, which recently has been proposed as a regulatory mechanism for protein activity [64]. Earlier studies showed that exogenous peroxynitrite did not cause cell death of soybean cells and *Arabidopsis* plants at concentrations up to 1 mM and 3 mM of ONOO⁻, respectively [31,65].

In the course of evolutionary changes plants have developed mechanisms facilitating avoidance or active control of adverse environmental conditions. Active defense responses of plants to stressors are typically executed on the basis of the stimulation of

intracellular signaling pathways, leading to the expression of target genes connected with mechanisms of adaptation/acclimation or active cell death. There is a possibility that cell death may be an essential event leading to the activation of tolerance response, ensuring plant survival under adverse environmental conditions [27]. The concept presented by Overmyer et al. [66] assumes that active cell death is required for an enhanced effectiveness of protective responses in neighboring cells or in order to generate a mobile long signal. To verify if PCD symptoms are linked with the generation of post-stress signaling molecules in parts of the plant distal from stress perception (from roots to leaves), we used another experimental design with 14-day old lupine seedlings at the stage of fully expanded leaves. As we found, Cd treatment at the roots level triggered an early NO generation in the elongation and differentiation zones of lupine roots, with concomitant and enhanced production of this molecule in leaves. A similar effect was observed in leaf discs gained from Cd-challenged *Arabidopsis* plants [23]. NO production was ascribed to the Cd translocation from roots to leaves, since the NO-dependent DAF-2T fluorescence was detected in cells in which Cd is accumulated preferentially, i.e. in the stomata and the trichomes on the leaf surface [23]. Interestingly, we observed earlier that the response to heavy metal applied to roots of 14-day old lupine seedlings was found in leaf tissue where no metal ions were detected [67]. The results tempting to speculate that the translocation of other unknown Cd-induced signals can trigger NO production in distal organs.

Under conditions applied in our study bio-imaging showed that Cd-induced NO-dependent fluorescence was localized mainly in the vascular bundles and in surrounding cells (Fig. 7(a)–(c)). The vascular tissue is able to produce NO, which is linked with signaling functions of this molecule during cell differentiation, senescence, plant response to salinity, pathogen and defense-related compounds [68–72]. According to Gauples et al. [72], the mechanism of distal signaling involves the production and transport of NO in the phloem. The hypothetical function of NO action in the vascular tissue could be to bind NO to enzymes, thereby modifying their activity, which, in turn, would induce signal synthesis or activation [72]. Moreover, an extensive body of evidence indicates the involvement of S-nitrosoglutathione (GSNO) in the phloem systemic signaling, leading to systemic acquired resistance (SAR) against pathogens [73].

It was suggested earlier that nitric oxide [74] might be engaged in root signaling in plants exposed to Cd. Our new finding presented in this study is that PCD symptoms instigated by Cd pollution might be engaged in root-to-leaves signaling, since the TUNEL-positive reaction of root cells (Fig. 9) was correlated with an over-accumulation of the main components of the plant defense machinery in leaves (Fig. 8, Supporting information Fig. S3). NO, H_2O_2 , and SA-mediated defense signaling partially overlap, since the SA positive feedback loop is essential for amplifying the distal signal in the upper zone of the plant. In view of our observations and according to Overmyer et al. [66], cell dying via PCD under the influence of Cd ions, might constitute an important element of the tolerance mechanism, increasing defense readiness of distal organs, not directly exposed to the metal. Obviously, if all root cells responded to Cd by the induction of PCD, the whole organ would quickly die [19]. Thus, we hypothesize that a specific threshold for triggering PCD exists and NO participates in the controlling of this threshold. As we have proved, only the tightly balanced level of NO with a concomitant NADPH-oxidase-dependent O_2^- generation upon a lower Cd stress was able to establish the threshold for PCD in lupine root seedlings.

In studies on NO we again face a situation when this molecule shows us the power of its multifaceted action, known as the Janus face of NO. On the one hand, NO enhances the accumulation of the

toxic metal and induces cell death, while on the other hand it is engaged in the generation or constitutes a distal signal that might help the plant to activate survival signaling in upper plant organs.

Acknowledgments

This work was supported by funds from Ministry of Science and Higher Education (project no. N N303303634 to E.A.G.).

Appendix A. Supporting information

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.plaphy.2012.06.018](https://doi.org/10.1016/j.plaphy.2012.06.018).

References

- [1] M. Souter, K. Lindsey, Polarity and signalling in plant embryogenesis, *J. Exp. Bot.* 51 (2000) 971–983.
- [2] L. Lombardi, S. Casani, N. Ceccarelli, L. Gallechi, P. Picciarelli, R. Lorenzi, Programmed cell death of the nucellus during *Sechium edule* Sw. seed development is associated with activation of caspase-like proteases, *J. Exp. Bot.* 58 (2007) 2949–2958.
- [3] A.H.L.A.N. Gunawardena, J.S. Greenwood, N.G. Dengler, Programmed cell death remodels lace plant leaf shape during development, *Plant Cell* 16 (2004) 60–73.
- [4] J. Floryszak-Wieczorek, M. Arasimowicz, G. Milczarek, H. Jeleń, H. Jackowiak, Only an early nitric oxide burst and the following wave of secondary nitric oxide generation enhanced effective defence responses of pelargonium to a necrotrophic pathogen, *New Phytol.* 175 (2007) 718–730.
- [5] H.P. Bais, R. Vepachedu, S. Gilroy, R.M. Callaway, J.M. Vivanco, Allelopathy and exotic plant invasion: from molecules and genes to species interactions, *Science* 301 (2003) 1377–1380.
- [6] M.C. Drew, C.J. He, P.W. Morgan, Programmed cell death and aerenchyma formation in roots, *Trends Plant Sci.* 5 (2000) 123–127.
- [7] M. Marsoni, C. Cantara, M.C. De Pinto, C. Gadaleta, L. De Gara, M. Bracale, C. Vannini, Exploring the soluble proteome of tobacco bright yellow-2 cells at the switch towards different cell fates in response to heat shocks, *Plant Cell Environ.* 33 (2010) 1161–1175.
- [8] C. Langebartels, H. Wohlgenuth, S. Kschieschan, S. Grün, H. Sandermann, Oxidative burst and cell death in ozone-exposed plants, *Plant Physiol. Biochem.* 40 (2002) 567–575.
- [9] L. Zhang, Q. Xu, D. Xing, C. Gao, H. Xiong, Real-time detection of caspase-3-like protease activation in vivo using fluorescence resonance energy transfer during plant programmed cell death induced by ultraviolet C overexposure, *Plant Physiol.* 150 (2009) 1773–1783.
- [10] S.J. Lin, Y. Wang, G.X. Wang, Salt stress-induced programmed cell death in tobacco protoplasts is mediated by reactive oxygen species and mitochondrial permeability transition pore status, *J. Plant Physiol.* 163 (2006) 731–739.
- [11] B.S.H. Behboodi, L. Samadi, Detection of apoptotic bodies and oligonucleosomal DNA fragments in cadmium-treated root apical cells of *Allium cepa* Linnaeus, *Plant Sci.* 167 (2004) 411–416.
- [12] E.T. Iakimova, E.J. Woltering, V.M. Kapchina-Toteva, F.J.M. Harren, S.M. Cristescu, Cadmium toxicity in cultured tomato cells – role of ethylene, proteases and oxidative stress in cell death signaling, *Cell Biol. Int.* 32 (2008) 1521–1529.
- [13] R. De Michele, E. Vurro, C. Rigo, A. Costa, L. Elvir, M. Di Valentin, M. Careri, M. Zottini, L. Sanita di Toppi, F.L. Schiavo, Nitric oxide is involved in cadmium-induced programmed cell death in *Arabidopsis* suspension cultures, *Plant Physiol.* 150 (2009) 217–228.
- [14] W. Ma, H. Xu, Y. Xu, Z. Chen, M. He Ma, Nitric oxide modulates cadmium influx during cadmium-induced programmed cell death in tobacco BY-2 cells, *Planta* 232 (2010) 325–335.
- [15] V.L. Souza, A.A. de Almeida, S.G. Lima, J.C. de M. Cascardo, D. da C. Silva, P.A. Mangabeira, F.P. Gomes, Morphophysiological responses and programmed cell death induced by cadmium in *Genipa americana* L. (Rubiaceae), *Biometals* 24 (2011) 59–71.
- [16] L. Sanità di Toppi, R. Gabbriellini, Response to cadmium in higher plants, *Environ. Exp. Bot.* 41 (1999) 105–130.
- [17] G. DalCorso, S. Farinati, A. Furini, Regulatory networks of cadmium stress in plants, *Plant Signal. Behav.* 5 (2010) 663–667.
- [18] M. Fojtová, A. Kovarik, Genotoxic effect of cadmium is associated with apoptotic changes in tobacco cells, *Plant Cell Environ.* 23 (2000) 531–537.
- [19] A. Kuthanova, L. Fischer, P. Nick, Z. Opatrny, Cell cycle phase-specific death response of tobacco BY-2 cell line to cadmium treatment, *Plant Cell Environ.* 31 (2008) 1634–1643.
- [20] A. Metwally, I. Finkemeier, M. Georgi, K.J. Dietz, Salicylic acid alleviates the cadmium toxicity in barley seedlings, *Plant Physiol.* 132 (2003) 272–281.
- [21] Y.T. Hsu, C.H. Kao, Abscisic acid accumulation and cadmium tolerance in rice seedlings, *Physiol. Plant.* 124 (2005) 71–80.
- [22] M. Rodríguez-Serrano, M.C. Romero-Puertas, D.M. Pazmino, P.S. Testillano, M.C. Risueno, L.A. del Rio, L.M. Sandalio, Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium, *Plant Physiol.* 150 (2009) 229–243.
- [23] A. Besson-Bard, A. Gravot, P. Richaud, P. Auroy, C. Duc, F. Gaymard, L. Tacconat, J.P. Renou, A. Pugin, D. Wendehenne, Nitric oxide contributes to cadmium toxicity in *Arabidopsis* by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake, *Plant Physiol.* 149 (2009) 1302–1315.
- [24] M. Leitner, E. Vandelle, F. Gaupels, D. Bellin, M. Delledonne, NO signals in the haze: nitric oxide signalling in plant defence, *Curr. Opin. Plant Biol.* 12 (2009) 451–458.
- [25] F.J. Corpas, M. Leterrier, R. Valderrama, M. Airaki, M. Chaki, J.M. Palma, J.B. Barroso, Nitric oxide imbalance provokes a nitrosative response in plants under abiotic stress, *Plant Sci.* 181 (2011) 604–611.
- [26] J.T. Hancock, S.J. Neill, I.D. Wilson, Nitric oxide and ABA in the control of plant function, *Plant Sci.* 181 (2011) 555–559.
- [27] M. Arasimowicz-Jelonek, J. Floryszak-Wieczorek, E.A. Gwóźdź, The message of nitric oxide in cadmium challenged plants, *Plant Sci.* 181 (2011) 612–620.
- [28] A. Besson-Bard, D. Wendehenne, NO contributes to cadmium toxicity in *Arabidopsis thaliana* by mediating an iron deprivation response, *Plant Signal. Behav.* 4 (2009) 252–254.
- [29] K. Valentovičová, L. Halušková, J. Huttová, I. Mistrik, L. Tamás, Effect of cadmium on diaphorase activity and nitric oxide production in barley root tips, *J. Plant Physiol.* 167 (2010) 10–14.
- [30] M.D. Groppa, E.P. Rosales, M.F. Iannone, M.P. Benavides, Nitric oxide, polyamines and Cd-induced phytotoxicity in wheat roots, *Phytochemistry* 69 (2008) 2609–2615.
- [31] M. Delledonne, J. Zeier, A. Marocco, C. Lamb, Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease response, *Proc. Natl. Acad. Sci. U.S.A.* 98 (2001) 13454–13459.
- [32] L.A.J. Mur, T.L.W. Carver, E. Prats, NO way to live; the various roles of nitric oxide in plant–pathogen interactions, *J. Exp. Bot.* 57 (2006) 489–505.
- [33] E. Zago, S. Morsa, J.F. Dat, P. Alard, A. Ferrarini, D. Inze, M. Delledonne, F. Van Breusegem, Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco, *Plant Physiol.* 141 (2006) 404–411.
- [34] M. Zottini, A. Costa, R. De Michele, F. Lo Schiavo, Role of nitric oxide in programmed cell death, in: S. Hayat, M. Mori, J. Pichtel, A. Ahmad (Eds.), *Nitric Oxide in Plant Physiology*, Wiley-VCH Verlag GmbH & Co. KGaA, 2010, pp. 77–87.
- [35] R. Przymsiński, R. Rucińska-Sobkowiak, B. Ilksa, E.A. Gwóźdź, Organospecific responses of lupin seedlings to lead. Localization of hydrogen peroxide and peroxidase activity, *Acta Physiol. Plant.* 29 (2007) 411–416.
- [36] J. Floryszak-Wieczorek, G. Milczarek, M. Arasimowicz, A. Ciszewski, Do nitric oxide donors mimic an endogenous NO-related response in plants? *Planta* 224 (2006) 1363–1372.
- [37] P.L. Olive, J.P. Banath, The comet assay: a method to measure DNA damage in individual cells, *Nat. Prot.* 1 (2006) 23–29.
- [38] R. Rucińska, R. Sobkowiak, E.A. Gwóźdź, Genotoxicity of lead in lupin root cells as evaluated by the comet assay, *Cell. Mol. Biol. Lett.* 9 (2004) 519–528.
- [39] N.O. Arita, M.F. Cohen, G. Tokuda, H. Yamasaki, Fluorometric detection of nitric oxide with diamino fluoresceins (DAFs): applications and limitations for plant NO research, in: L. Lamattina, J.C. Polacco (Eds.), *Nitric Oxide in Plant Growth, Development and Stress Physiology*, Springer, 2007, pp. 268–280.
- [40] M. Sagi, R. Fluhr, Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection, *Plant Physiol.* 126 (2001) 1281–1290.
- [41] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [42] N. Doke, Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race *Phytophthora infestans* and to the hyphal wall components, *Physiol. Plant Pathol.* 23 (1983) 345–357.
- [43] M. Becana, P. Aparicio-Tejo, J.J. Irigoyen, M. Sanchez-Diaz, Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*, *Plant Physiol.* 82 (1986) 1169–1171.
- [44] H. Thordal-Christensen, Z. Zhang, Y. Wei, D.B. Collinge, Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction, *Plant J.* 11 (1997) 1187–1194.
- [45] J.C. Huang, D.J. Li, J.C. Diao, J. Hou, J.L. Yuan, G.L. Zou, A novel fluorescent method for determination of peroxynitrite using folic acid as a probe, *Talanta* 72 (2007) 1283–1287.
- [46] L. Ederli, L. Reale, L. Madoe, F. Ferranti, C. Gehring, M. Fornaciari, B. Romano, S. Pasqualini, NO release by nitric oxide donors in vitro and in planta, *Plant Physiol. Biochem.* 47 (2009) 42–48.
- [47] I.V. Seregin, V.B. Ivanov, Histochemical investigation of cadmium and lead distribution in plants, *Russ. J. Plant Physiol.* 44 (1997) 791–796.
- [48] P. Meuwly, J.P. Métraux, *Ortho*-anisic acid as internal standard for the simultaneous quantification of salicylic acid and its putative biosynthetic precursors in cucumber leaves, *Anal. Biochem.* 214 (1993) 500–505.

- [49] E.A. Gwóźdź, R. Przymusiński, R. Rucińska, J. Deckert, Plant cell responses to heavy metals: molecular and physiological aspects, *Acta Physiol. Plant.* 19 (1997) 459–465.
- [50] A. Balestrazzi, A. Macovei, C. Testoni, E. Raimondi, M. Donà, D. Carbonera, Nitric oxide biosynthesis in white poplar (*Populus alba* L.) suspension cultures challenged with heavy metals, *Plant Stress* 3 (2009) 1–6.
- [51] M.L. Lanteri, G.C. Pagnussat, L. Lamattina, Calcium and calcium-dependent protein kinases are involved in nitric oxide- and auxin-induced adventitious root formation in cucumber, *J. Exp. Bot.* 57 (2006) 1341–1351.
- [52] J. Xiong, H. Lu, K. Lu, Y. Duan, L. An, C. Zhu, Cadmium decreases crown root number by decreasing endogenous nitric oxide, which is indispensable for crown root primordia initiation in rice seedlings, *Planta* 230 (2009) 599–610.
- [53] J. Xu, W. Wang, H. Yin, X. Liu, H. Sun, Q. Mi, Exogenous nitric oxide improves antioxidative capacity and reduces auxin degradation in roots of *Medicago truncatula* seedlings under cadmium stress, *Plant Soil* 326 (2010) 321–330.
- [54] M. Rodriguez-Serrano, M.C. Romero-Puertas, A. Zabalza, F.J. Corpas, M. Gomez, L.A. del Rio, L.M. Sandalio, Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo, *Plant Cell Environ.* 29 (2006) 1532–1544.
- [55] L. Tamás, K. Valentovicová, L. Halušková, J. Huttová, I. Mistrík, Effect of cadmium on the distribution of hydroxyl radical, superoxide and hydrogen peroxide in barley root tip, *Protoplasma* 236 (2009) 67–72.
- [56] E. Olmos, J.R. Martínez-Solano, A. Piqueras, E. Hellín, Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line), *J. Exp. Bot.* 54 (2003) 291–301.
- [57] L. Garnier, F. Simon-Plas, P. Thuleau, J.P. Agnel, J.P. Blein, R. Ranjeva, J.L. Montillet, Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity, *Plant Cell Environ.* 29 (2006) 1956–1969.
- [58] B. Belenghi, F. Acconcia, M. Trovato, M. Perazolli, A. Bocedi, F. Policelli, P. Ascenzi, M. Delledonne, AtCYS1, a cystatin from *Arabidopsis thaliana*, suppresses hypersensitive cell death, *Eur. J. Biochem.* 270 (2003) 2593–2604.
- [59] J. Zhao, K. Fujita, K. Sakai, Reactive oxygen species, nitric oxide, and their interactions play different roles in *Cupressus lusitanica* cell death and phytoalexin biosynthesis, *New Phytol.* 175 (2007) 215–229.
- [60] L. Lombardi, N. Ceccarelli, P. Picciarelli, C. Sorcea, R. Lorenzia, Nitric oxide and hydrogen peroxide involvement during programmed cell death of *Sechium edule* nucellus, *Physiol. Plant.* 140 (2010) 89–102.
- [61] M. Delledonne, Y. Xia, R.A. Dixon, C. Lamb, Nitric oxide functions as a signal in plant disease resistance, *Nature* 394 (1998) 585–588.
- [62] M.C. de Pinto, F. Tommasi, L. De Gara, Changes in the antioxidant systems as part of the signalling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco BY-2 cells, *Plant Physiol.* 130 (2002) 698–708.
- [63] N. Lehotai, A. Pető, S. Bajkán, S. Erdei, I. Tari, Z. Kolbert, In vivo and in situ visualization of early physiological events induced by heavy metals in pea root meristem, *Acta Physiol. Plant.* 33 (2011) 2199–2207.
- [64] M. Arasimowicz-Jelonek, J. Floryszak-Wieczorek, Understanding the fate of peroxynitrite in plant cells – from physiology to pathophysiology, *Phytochemistry* 72 (2011) 681–688.
- [65] M.C. Romero-Puertas, M. Laxa, A. Matte, F. Zaninotto, I. Finkemeier, A.M. Jones, M. Perazolli, E. Vandelle, K.J. Dietz, M. Delledonne, S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration, *Plant Cell* 19 (2007) 4120–4130.
- [66] K. Overmyer, M. Brosché, J. Kangasjärvi, Reactive oxygen species and hormonal control of cell death, *Trends Plant Sci.* 8 (2003) 335–342.
- [67] R. Przymusiński, A. Banaszak, E.A. Gwóźdź, Organospecific responses of lupin seedlings to lead. I. Localization of lead ions and stress proteins, *Acta Physiol. Plant.* 23 (2001) 109–116.
- [68] F.J. Corpas, J.B. Barroso, A. Carreras, M. Quiros, A.M. Leon, M.C. Romero-Puertas, F.J. Esteban, R. Valderrama, J.M. Palma, L.M. Sandalio, M. Gomez, L.A. del Rio, Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants, *Plant Physiol.* 136 (2004) 2722–2733.
- [69] C. Gabaldon, L.V. Gomez-Ros, M.A. Pedreno, A.R. Barcelo, Nitric oxide production by differentiating xylem of *Zinnia elegans*, *New Phytol.* 165 (2005) 121–130.
- [70] M.E. Requena, C. Egea-Gilabert, M.E. Candela, Nitric oxide generation during the interaction with *Phytophthora capsici* of two *Capsicum annuum* varieties showing different degrees of sensitivity, *Physiol. Plant.* 24 (2005) 50–60.
- [71] R. Valderrama, F.J. Corpas, A. Carreras, A. Fernández-Ocaña, M. Chaki, F. Luque, M.V. Gomez-Rodriguez, P. Colmenero-Varea, L.A. del Rio, J.B. Barroso, Nitrosative stress in plants, *FEBS Lett.* 581 (2007) 453–461.
- [72] F. Gaupels, A.C.U. Furch, T. Will, L.A.J. Mur, K.H. Kogel, A.J.E. Bel, Nitric oxide generation in *Vicia faba* phloem cells reveals them to be sensitive detectors as well as possible systemic transducers of stress signals, *New Phytol.* 178 (2008) 634–646.
- [73] A.C. Vlot, D.F. Klessig, S.W. Park, Systemic acquired resistance: the elusive signal(s), *Curr. Opin. Plant Biol.* 11 (2008) 436–442.
- [74] M. Kopyra, E.A. Gwóźdź, Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*, *Plant Physiol. Biochem.* 41 (2003) 1011–1017.